

SUBSTRATE MEASURING METHOD AND DEVICE

BACKGROUND OF THE INVENTION

Field of the Invention

5 This invention relates to a method and device for making measurements on the surface of a substrate, in particular, to a method and device for making measurements for portions to be measured on a substrate using scan operation (scanning).

10 Related Background Art

 In recent years, with the development of biotechnology, there have been increasing needs for making measurements for and detection of the samples from living organisms. The samples from living
15 organisms are, in general, often hard to carry out a close measurement, unlike chemical substances obtained using the technique of synthetic chemistry, because

1. they has extremely wide variegation;
2. their absolute quantity is small; and
- 20 3. those having similar physical properties must be distinguished.

 To date there have been devised various methods of making measurements for such samples; and among them, the measurements using a solid phase substrate are
25 receiving most attention now. The measuring technique using a solid phase substrate is, for example, such that it allows detection probes (for example, antibody)

fixed on or absorbed into a substrate to react with a sample labeled with a fluorescent substance (for example, antigen) on the substrate and makes measurements for the sample by observing the
5 fluorescence on the substrate.

The principal reasons (advantages) that this measuring technique attracts particular attention are, for example, as follows:

1. measurements can be performed for an infinitely
10 small amount of sample by decreasing the amount (area) of the detection probes attached on the substrate, and hence, the absolute quantity of the sample is not required to be large;

2. measurements of multiple items can be performed
15 simultaneously by arraying various detection substances on the substrate; and

3. the substrate is easy to handle because it is not in a liquid phase, but in a solid phase.

The measurements for a sample using a solid-phase
20 substrate have been applied, for example, to the detection of the base sequences of nucleic acids. In such a detection operation, first, various types of single-stranded DNAs (DNA probes) with various base sequences are attached on a substrate in an array and
25 then a DNA labeled with, for example, fluorescent pigment is applied thereto. When there exists in the sample a sequence complementary to the DNA probe on the

substrate, the fluorescent substance is adsorbed on the substrate (hybridization); thus, the base sequence contained in the sample can be examined by checking the state of hybridization against the DNA probe on the substrate. As a matter of fact, Affymetrix in the U.S has developed a DNA chip such that as many as about 10,000 types of DNA probes are arrayed on its micro-area by the photolithographic process and has applied the same to the analysis of DNA base sequences.

10 An area on which each probe is attached in an array consists of extreme-micro-areas. The reason for this is that, when the absolute quantity of the sample is small, a highly sensitive detection is made possible by concentrating the sample on a micro-probe-area so as to allow the amount of sample per unit to be larger, that is, to provide higher sample density.

Scattering the sample on a wide-probe-area lowers a detection sensitivity.

20 Accordingly, detectors for use in the observation of such arrays are often microscopes or devices including a confocal optical system. In such cases, the whole of the array is not subjected to analyzing and measuring processing, but it needs to be divided into micro-areas and subjected to read processing area by area with a microscope with high magnification. In other words, detectors involving scan operation are needed. Scanning methods include, for example, methods

to perform scan operation by moving a light detecting portion over a substrate having certain dimensions and by moving a substrate while fixing a light detecting portion. Thus various types detectors have been
5 designed so as to match various types scanning methods.

For example, an exclusive detector is commercially available from Hewlet Packard which is a device for making measurements for the DNA microarrays by Affymetrix, and this device consists of both a cofocal
10 optical system and a scanner.

In scanners in common use, for example when the substrate to be measured is rectangular, read operation is performed by scanning from one end of the substrate linearly in a fixed direction to the other end and then
15 returning so as to scan the next area, and repeating this motion. In other words, the scan operation involves a reciprocating motion in terms of its scanning direction (scan operation is not performed in one direction, but involves a returning motion).

20 However, scan operation involving a reciprocating motion gives rise to problems of making the control and construction of the device more complicated and requiring a longer measuring period. The longer measuring period gives rise to another problem of
25 creating a difference in measuring conditions between the areas of the first measurement and the last measurement. In order to prevent this problem, a

special device needs to be installed. Further, detection is performed using fluorescent pigments, the problems of color fading and denaturation may occur depending on the pigment.

5 A discontinuous scan operation involving a returning motion attendant to a reciprocating motion is likely to cause a decrease in positional accuracy compared with a continuous scan operation. This may affect the measurements on the substrate consisting of
10 high-density microarrays.

SUMMARY OF THE INVENTION

Accordingly, the object of the present invention is to solve the problems attendant to the above
15 scanning method.

Specifically, an object of the present invention is to provide a method of making measurements for the sample on the measuring surface of the substrate, comprising forming a circular orbit of detection areas
20 on the measuring surface of the substrate by moving the area to be detected with a detector relative to the substrate.

One example of the measuring methods described above is such that the measurements for the sample are
25 made while forming a rotational surface of the subject and the circular orbit as described above by rotating the substrate around the axis perpendicular to the

surface of the substrate on which measurements are made, in particular, the measurements are made by moving the detection area relative to the rotational surface of the substrate on which measurements are made.

Another example of the measuring methods described above is such that it performs measurements while forming the circular orbit of the measuring areas as described above by allowing the detector to perform a rotational motion.

The sample described above is fixed on, adsorbed into, or trapped in the substrate as described above and it is, for example, specifically fixed on the substrate by the probes arranged on the surface of the same.

Preferably, the probes and the sample on the substrate as described above are DNA, protein and peptide nucleic acid (PNA). The measurements for the above sample are made using luminescence such as fluorescence and chemilluminescence.

Specifically, when making measurements for the sample, any one of the absorption, transmission and reflection of the incident light to said sample is measured.

When multiple labels are detected with multiple detectors in the measurements for the above sample, the multiple labels can be detected with their

corresponding detectors simultaneously.

Another object of the present invention is to provide a device for making measurements for a sample on the measuring surface of the substrate, comprising:

5 a detector for measuring for a label from the sample;

 a means for supporting the substrate having the sample as the subject of measurements on its measuring surface; and

10 a means for forming a circular orbit of detection areas on the measuring surface of the substrate by moving the detection areas, in which detection is performed with a detector, relative to the substrate.

15 BRIEF DESCRIPTION OF THE DRAWINGS

 FIG. 1 is a view of a substrate on which 4 types of previously prepared DNA are printed;

 FIG. 2 is a view of a substrate measuring device;

 FIG. 3 is an enlarged view of the stage portion of
20 a substrate measuring device; and

 FIG. 4 is a view showing the scanning process in measuring areas.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

25 The substrate measuring method and device of the present invention are characterized in that they allow a substrate, as a subject of measurements, to rotate

around a certain point in a plane in which the substrate is placed and scan the measuring area of the substrate by the use of the rotational movement thereof.

5 The use of the rotational movement of the substrate allows the scan operation to be performed in a fixed direction while omitting the reciprocating scan operation involving stopping scanning and changing the scanning direction, which are likely to affect the
10 accuracy of the measurement, resulting in improvement in the accuracy and speed of measurement.

 As a driving system used for making measurements for the areas on the substrate, the method and device require a driving system for rotating the substrate.
15 If measurements cannot be made for all the measuring areas by the use of the rotational movement of the substrate alone, an additional driving system for changing the radial length, from the center of the rotation to the measuring portion, is optionally
20 installed, so as to enable the measurements for all the measuring areas.

 From the standpoint of easy rotation, the substrate is desirably a special substrate which incorporates a central axis of rotation as a
25 constituent, so that the rotational driving of the axis can be directly transmitted thereto, like data disks commonly used in daily life; however, if it is

impossible to prepare such a substrate, the substrate
may be rotated using a holder on which it is fixed.
When the central axis of rotation cannot be
incorporated in the substrate plane for the reasons
5 related to measurement, the substrate may be rotated
around the central axis established outside it while
being fixed on the holder.

Generally, it is often easier to move the
substrate than to move the measuring portions because
10 of the structure of the devices; however, in cases
where the substrate is difficult to rotate, a driving
system may be used which does not rotate the substrate,
but rotates mainly the measuring portions.

In cases where measurements need to be made for
15 multiple items such as absorption and fluorescence, the
measuring portions can be provided item by item and
measurements can be performed for all the portions
simultaneously.

Measurements may be made at suitable intervals
20 while allowing the measuring portions to continuously
move at a constant speed, or they may be made while
stopping the substrate intermittently after allowing it
to move by a predetermined distance. When rotating the
substrate, desirably the rotational speed is variable
25 so that it can be properly established depending on the
necessary measuring time and resolution. When the
accuracy and reliability of the data taken on a

measuring portion of the substrate can be improved by
integrating the measured values obtained from multiple
times of measurement on the same portion, such
integration may be carried out according to the
5 situation.

The data obtained from the measurements on the
measuring portions become final data in combination
with, for example, the positional information obtained
from the rotation angle of the substrate and the
10 distance from the central axis of the rotation
(radius). And in order to obtain more accurate
positional information, desirably the measurements are
performed while picking up the movements of some
markers having been previously provided on or around
15 the substrate. This enables measurements with a higher
accuracy.

The method of measuring fluorescence intensity in
accordance with the present invention is applicable to
the detection of substances using a solid-phase
20 substrate, to which increasing attention has been being
paid in recent years. One example of the applications
is the DNA chips developed by Affymetrix in the U.S.,
as described above. The substrate measuring method of
the present invention can be used as a scanner for
25 measuring the fluorescence or luminescence intensity on
the solid-phase substrates on which various types of
probes such as DNA and proteins are arrayed in high

density.

Example 1

(1) Preparation of Substrate

A silica glass substrate 3 cm in diameter and 0.5
5 mm in thickness was prepared. In order to allow the
substrate to rotate, a structure capable of
transmitting rotational drive was provided for the
substrate on the circular portion with its center at
the center of the substrate and with a diameter of 1
10 cm.

The silica glass substrate was water-washed
lightly, and then subjected to ultrasonic cleaning for
20 minutes while being immersed in an exclusive
substrate cleaning solution, and left stand one whole
15 day and night. Then the substrate was taken from the
cleaning solution so as to wash the cleaning solution
away with water and deionized water, and immersed in 1
M NaOH aqueous solution having been heated to 60°C for
20 minutes. The substrate was taken from the NaOH
20 aqueous solution, so as to wash the NaOH aqueous
solution away with water and ultrapure water, and
subjected to ultrasonic cleaning in ultrapure water for
20 minutes.

Then the substrate was immersed in a silane
25 coupling agent (manufactured by Shin-Etsu Chemical Co.,
Ltd., brand name: KBM 603) for one hour, which was
previously dissolved in water to be 1% aqueous solution

and subjected to hydrolysis for about one hour. The substrate was washed lightly with ultrapure water, dried by removing water droplets remaining on the surface with nitrogen gas, and baked in an oven at
5 120° C for 2 hours. Amino groups were introduced to the surface of the glass substrate by combining the silane coupling agent with the same.

Then a crosslinking agent, EMCS (N-(Maleimidocaproyloxy) succinimide), manufactured by
10 Dojindo Laboratories was dissolved in a mixed solvent (ethanol : DMSO = 1 : 1) in the proportion of 3 mg to 10 ml. The glass having been baked previously was immersed in the obtained EMCS solution and left for 2 hours. After taking the substrate from the EMCS
15 solution and washing lightly with the same mixed solvent as above, the droplets remaining on the surface of the substrate was subjected to substitution into ethanol, and the substrate was dried by removing the droplets with nitrogen gas. Thus obtained was a
20 substrate (EMCS substrate) with the entire surface (the surface of both sides) of which EMCS was combined. EMCS contains a succinimide group and a maleimido group; and since the succinimide group ?????? the amino group on the surface of the substrate, the surface of
25 the substrate had the maleimido group having been introduced thereon.

(2) DNA Attachment

Modified 18 mer DNAs (probes) with a thiol group (SH group) attached to one terminal thereof were synthesized by BEX at the request of the applicants of this invention. The SH group was attached to each 5' terminal and the DNA base sequences were as follows:

No. 1: 5'HS-ACTGGCCGTCGTTTTACA³'

(SEQ. ID No. 1)

No. 2: 5'HS-ACTGGCCGTTGTTTTACA³'

(SEQ. ID No. 2)

10 No. 3: 5'HS-ACTGGCCGCTTTTTTTACA³'

(SEQ. ID No. 3)

No. 4: 5'HS-ACTGGCATCTTGTTTACA³'

(SEQ. ID No. 4)

The above DNAs were dissolved in SG Clear (aqueous solution containing 7.5% of glycerol, 7.5% of urea, 7.5% of thiodiglycol and 1% of Acetylenol EH (manufactured by Kawamura Fine Chemicals)), which is a solvent for use in thermal jet printer (Bubble jet printer = BJ printer), and adjusted to a final concentration of 8 μ M. And a cartridge for use in ink jet printers is filled with the DNA solution. The four types of probes are arranged in the pattern shown in FIG. 1. Each type of probe consists of an area 2 of 5 mm \times 10 mm, and are arranged each area a probe 3 of the same type in 120 dpi density.

Then the substrate with the DNA solution placed thereon was left in a humidifying chamber for 30

minutes to react the substrate with DNA.

The BJ printer used was a remodeled type of BJ printer BJC-600, which is manufactured by Canon Inc. and capable of doing litho printing, and the amount of
5 the DNA solution discharged per dot was 24 picoliters.

After completion of the reaction, the substrate was rinsed with 1M NaCl/50 mM phosphate buffer solution (pH 7.0), so as to wash the DNA solution on the surface of the glass away completely. Then the substrate was
10 immersed in 2% bovine serum albumin aqueous solution, left for 2 hours, and subjected to blocking reaction. After the blocking reaction, the substrate was again rinsed with 1M NaCl/50 mM phosphate buffer solution (pH 7.0), so as to obtain the substrate to which DNAs were
15 attached.

(3) Hybridization

A DNA labeled with Rhodamine was synthesized which had Rhodamine having been attached to 5' terminal thereof and had a complementary sequence to the probe
20 No. 1. This synthesis was also carried out by BEX at the request of the applicants of this invention. This labeled DNA was dissolved in 1M NaCl/50 mM phosphate buffer solution (pH 7.0) so as to obtain a final concentration of 1 μ M, and 2 ml of the obtained
25 solution was sealed into a hybridization package together with the substrate, so as to subject them to hybridization reaction for 3 hours.

Then the substrate 1 was rinsed with 1M NaCl/50 mM phosphate buffer solution (pH 7.0) to obtain the substrate as the subject of measurements.

(4) Device Construction

5 The stage portion of a fluorescence microscope manufactured by Nikon Corporation was remodeled, and a stage 4 shown in FIG. 2 was fixed to the fluorescence microscope. The stage 4 consisted of a rotational drive stage 4a for rotating the substrate 1 and a
10 horizontal drive stage 4b for allowing the same to move horizontally. FIG. 3 shows the details of the stage portion. The stage portion was remodeled so that any desired portion of the measuring areas on the substrate could be set under the object lens 7 of the
15 fluorescence microscope 5 by properly controlling the two driving devices.

 The two driving devices were designed in such a manner that they could output their respective positional information to an image processing apparatus
20 9 and the image processing apparatus could pick up the positions on the substrate for which measurements were being made with the lens.

 For the light source 6 of the fluorescence microscope, a mercury lamp in common use was used. A
25 filter and a dichroic mirror were configured in such a manner that the wave lengths of excitation light and fluorescence were from 455 nm to 595 nm and from 610 nm

to 725 nm, respectively. The fluorescence obtained was output into the image processing apparatus via a detector 8 and subjected to proper image processing in combination with the positional information output from the stage. In other words, the entire image of the measuring areas on the substrate could be displayed by superimposing the images obtained from the detector based on the positional information sent from the stage.

10 (5) Substrate Measurement

After fixing the substrate 7 to the holder of the stage and bringing the same into focus, the two driving devices were started so as to perform measurements. The scanning method was established so that scanning could be performed from the periphery of the substrate spirally inward by decreasing the spacing between the central axis of the substrate and the object lens while allowing the substrate to rotate clockwise (refer to FIG. 4). In order to make possible the measurements for all the measuring areas on the substrate by allowing the detector to scan the measuring areas 10, the two driving devices were properly controlled, in addition, the rotational speed of the disk was set for the CLV method (constant linear velocity method) so as to uniform the reading accuracy.

For the object lens, a lens of 40 magnifications was used, and it was set in such a manner that one spot

became a circle with a diameter 5 μm . The rotational speed was set in such a manner that the liner velocity was always 500 mm/s and controlled so that the spacing between the rotational axis and the object lens was
5 decreased by 5 μm each time the disk made one rotation.

For the detector, a photomultiplier tube equipped with a barrier filter was used. The signal detected was sent to the image processing apparatus as an electric signal of one dimension, and the data was
10 processed in combination with the positional information from the disk.

After the measurements with the image processing apparatus, images were obtained which were nearly equivalent to those expected from the print patterns and the sequences of the probes. Specifically, in the
15 area of No. 1 where the sequences of the probe and the sample were completely complementary to each other, intensive fluorescence was detected. In the areas of No. 2 and No. 3, less intensive fluorescence patterns
20 were obtained, and in the area of No. 4, almost no fluorescence was detected.

The patterns obtained from the areas of No. 1, No. 2 and No. 3 were in accord with the print patterns when preparing the substrate.

25 The determination of the average intensity of fluorescence for each probe on the image processing apparatus showed that, in the probe No. 1 having a

sequence completely matching that of the labeled DNA,
the fluorescence yield was 4600, and in the probe No. 2
of which sequence had one mismatched base, the
fluorescence yield was 2800. Further, in the probe No.
5 3 of which sequence had 3 mismatched bases, the
fluorescence yield was as small as 2100, which was less
than half of the fluorescence yield of the completely
matched sequence. In the probe No. 4 of which sequence
had 6 mismatched bases, fluorescence was hardly
10 observed.

As described above, substrate measurements could
be performed accurately and quantitatively using the
method in accordance with the present invention.

The substrate measuring method in accordance with
15 the present invention offers the advantage that
substrate measurements can be performed more easily
while omitting complicated scan operation. Since scan
operation for making measurements for the portions on a
substrate are performed by rotating the substrate,
20 driving systems for moving the substrate can be
simplified. Furthermore, the measuring method in
accordance with the present invention enables the
continuous scan operation in which no return motion is
required, which results in high-speed and high-accuracy
25 measurements compared with those of the methods
performing discontinuous scan operation.

Sequence Listing

SEQ. ID No.: 1

Length: 18

Type: Nucleic Acid

5 Strandedness: Single Strand

Topology: Linear

Molecule type: Other Nucleic Acid, Synthetic DNA

Sequence

ACTGGCCGTC GTTTTACA

10 18

SEQ. ID No.: 2

Length: 18

Type: Nucleic Acid

15 Strandedness: Single Strand

Topology: Linear

Molecule type: Other Nucleic Acid, Synthetic DNA

Sequence

ACTGGCCGTT GTTTTACA

20 18

SEQ. ID No.: 3

Length: 18

Type: Nucleic Acid

25 Strandedness: Single Strand

Topology: Linear

Molecule type: Other Nucleic Acid, Synthetic DNA

Sequence

ACTGGCCGCT TTTTACA

18

5 SEQ. ID No.: 4

Length: 18

Type: Nucleic Acid

Strandedness: Single Strand

Topology: Linear

10 Molecule type: Other Nucleic Acid, Synthetic DNA

Sequence

ACTGGCATCT TGTTTACA

18